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(54) Title: STABILIZED CONJUGATES OF UNCOMPLEXED SUBUNITS OF MULTIMERIC PROTEINS			
(57) Abstract			
<p>The present invention provides methods for preparing, and compositions comprising, stabilized protein-polymer conjugates. More particularly, the present invention relates to the stabilization of individual subunits of multisubunit proteins complexes by conjugation to polymers. Such conjugation acts to stabilize the individual subunit in its native conformation in liquid medium, which in turn acts to stabilize its biological activity.</p>			

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## STABILIZED CONJUGATES OF UNCOMPLEXED SUBUNITS OF MULTIMERIC PROTEINS

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### RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Serial No. 08/730,111, filed October 15, 1996, the contents of which are hereby incorporated by reference into the present disclosure.

### TECHNICAL FIELD

The present invention relates to the conjugation of uncomplexed subunits of multisubunit protein complexes with polymers to stabilize their conformation. More specifically, the present invention describes a method of stabilizing these individual subunits via covalent conjugation to a natural or synthetic polymer. The present invention also relates to stabilized conjugates of cardiac troponin I and stabilized conjugates of cardiac troponin T, and methods for their preparation.

### BACKGROUND ART

Proteins are composed of long chains of amino acids. The structure of proteins can be considered on four different levels. The primary structure refers to the specific order of amino acids in the polymer chain. The secondary structure refers to the interactions among and between the amino acids in the chain to form such structures as  $\alpha$  helices and  $\beta$  pleated sheets. The tertiary structure refers to the three-dimensional structure of the protein, which is also referred to as a protein's conformation. The quaternary structure refers to the spatial arrangement of individual polypeptides or "subunits" of multisubunit proteins.

The native conformation of a protein is only marginally stable. Thus, many proteins which are removed from their native environment and purified undergo

conformational changes which can cause a loss of biological activity, such as enzyme activity or antibody binding capacity. In particular, the individual (uncomplexed) subunits of multisubunit protein complexes may undergo dramatic conformational changes when separated from the other subunits of the complex and stored in a liquid medium.

5 It is often desirable to separate the individual subunits of a multisubunit protein complex, for example to study or exploit the biological activity of each individual subunit. However, this may not be possible if the individual subunits undergo conformational changes in their uncomplexed state that alter their biological activity. Accordingly, it is an object of the present invention to provide a method for stabilizing individual subunits of 10 multisubunit protein complexes.

15 Troponin is an example of a multisubunit protein complex which consists of three individual subunits; troponin T, troponin C and troponin I. The troponin complex is involved in the calcium-sensitive switch that regulates the interaction of actin and myosin in striated muscles. Troponin T binds the troponin complex to tropomyosin, while troponin I is the inhibitory subunit of the complex. Whereas troponin C from skeletal muscle and cardiac muscle is identical, troponin I and T from these two sources exist as different isoforms, each having a different amino acid sequences and thus a unique structure. Thus, 20 cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are of particular interest as cardiospecific markers.

25 After myocardial infarction, cTnT levels increase and remain elevated for an extended period. However, it has been reported that in a variety of disease states, cTnT is also expressed in skeletal muscle, which contributes to a lack of cardiospecificity of this protein. Furthermore, uremia, a condition associated with cardiomyopathy, is associated with elevated cTnT. Thus, a lack of absolute cardiospecificity makes this marker less than optimal for use in the early diagnosis of acute myocardial infarction (AMI).

Cardiac TnI is also released after acute myocardial infarction. In contrast to cTnT, cTnI has never been found in a healthy population, which includes marathon runners, in people with skeletal disease, or in patients undergoing non-cardiac operations. Thus, cTnI is a more specific marker for the diagnosis of AMI than other serum proteins.

30 A variety of immunoassays have been developed that utilize antibodies that can distinguish between the three troponins, and also between their different isoforms.

Monoclonal and polyclonal antibodies have been designed and used in immunoassays which can detect the cardiac-specific epitopes formed by the unique amino acid sequence of cTnI. See, for example PCT Patent Application No. WO 96/10076; European Patent No. 394,819 B1; and Adams *et al.*, *Circulation* 88:101-106 (1993). Larue *et al.*, (*Clin. Chem.* 39:972-979 (1993)) describe an immunoenzyme assay that is capable of detecting cTnI in the concentration range of 0.2 to 20 µg/L in 30 minutes.

5 Immunoassays have also been described which are specific for TnT. See, for example, Katus, *et al.*, *Circulation*, 83(3):902-912 (1991). An immunoassay for TnT is also commercially available from Boehringer Mannheim Corp., Indianapolis, IN.

10 Most immunoassays are designed to determine the concentration of a given marker in a patient's serum by comparing immunoassay results with the patient's serum to those obtained with control reagents of known concentration. One limitation in the development of immunoassays for the troponins involves the instability of the troponins in their uncomplexed state. Accordingly, there is a need for stabilized compositions of cTnI and  
15 cTnT that can be stored for extended periods of time, while retaining antibody binding capacity for use as control reagents in cTnI and cTnT immunoassays.

20 Stabilization of proteins via covalent conjugation to various polymers has been described. See, for example, U.S. Patent No. 4,902,502; U.S. Patent No. 5,468,478; U.S. Patent No. 4,806,524; Katre *et al.*, *J. Immunol.* 144:209-213 (1990); Abuchowski *et al.*, *J. Biol. Chem.* 252:3582-3586 (1977). The properties conferred on the conjugated protein have been cited as increased *in vivo* half life, increased stability in solution, increased solubility, decreased susceptibility to proteases and decreased immunogenicity and antigenicity. For example, Nitecki *et al.* (U.S. Patent No. 5,089,261) describe conjugating interleukin-2 to polyethylene glycol (PEG) to reduce immunogenicity.

25 In addition to stabilization of proteins via covalent conjugations to polymers, it has also been demonstrated that synthetic polymers are capable of providing a stabilizing effect via ionic interaction with proteins. For example, Marsh and Danielson (*Analyst* 120:1091-1096 (1995)) have described that the addition of PEG to an aqueous solutions of the multisubunit enzyme lactate dehydrogenase enhances the ability of the enzyme subunits to remain complexed.

5

The present invention relates to the finding that covalent conjugation of individual protein subunits of multisubunit proteins to polymers stabilizes the protein subunit, *i.e.* it maintains its native conformation in a liquid medium for a longer period of time than the equivalent unconjugated individual subunit. In a preferred embodiment, the present invention also relates to cTnI-polymer conjugates and cTnT-polymer conjugates which exhibit stabilized antibody binding capacity.

#### DISCLOSURE OF THE INVENTION

The present invention concerns stabilization of individual subunits of multisubunit protein complexes. Stabilization is accomplished by conjugating the individual subunits to a polymer. The stabilizing effect of conjugation allows the individual subunit to be stored in liquid medium for longer periods of time than an equivalent unconjugated or "free" individual subunit. This greatly enhances the shelf life of the composition. In a preferred embodiment, the stabilized individual subunits are either cTnI or cTnT, which in their unconjugated form are highly unstable in liquid medium.

15

Polymers which are useful in the present invention can be naturally occurring or synthetic. Whereas certain synthetic polymers may be preferred for stabilization of free cTnI, as will be discussed below, natural polymers such as serum proteins are preferred for stabilization of cTnT. A particularly preferred class of synthetic polymer is PEG. Other suitable polymers include, but are not limited to polyalkylene glycols, polyoxyethylated polyols, polyvinylpyrrolidone, polyhydroxyethyl methacrylate, polyvinyl alcohols, and polyurethane.

20

The polymers which are useful in the present invention may vary in molecular weight, and must have a molecular weight which is sufficient to stabilize the individual subunit. This generally requires that the polymer have a molecular weight between 100 and 200,000, more preferably between 1,000 and 40,000, and most preferably between 2,500 and 10,000.

25

In order to conjugate the polymer to the individual protein subunit, it should be in an "active" form, which means it must contain at least one reactive group capable of reacting with pendant groups on the protein to form a covalent linkage. When the polymer is PEG, a preferred active form is monomethoxy-PEG p-nitrophenyl carbonate.

The ratio of individual subunit to polymer in the conjugation reaction must be sufficient to stabilize the individual subunit. This generally requires that the polymer is provided in a molar concentration which is at least equivalent to the molar concentration of the individual subunit. Preferably, the polymer is provided in excess to ensure that a sufficient number of polymers are covalently attached to the individual subunits.

As an alternative to polymers, monomers (at least some of which are in an active form) can be used to form the protein-polymer conjugates of the present invention, which may polymerize during conjugation and may even attach directly to the protein subunit to afford the desired stability.

Another aspect of the present invention relates to compositions that consist of individual subunit-polymer conjugates in liquid medium. Suitable liquid media include water, aqueous solvents, serum, and mixtures thereof. Preferably, the liquid medium is mammalian serum, and more preferably, it is a mixture of human serum and bovine serum.

Other excipients, such as salts, buffers, proteins, polymers, carbohydrates, preservatives and reducing agents may also be added to the liquid medium.

A preferred embodiment of the present invention relates to stabilized conjugates of cTnI which are useful as control reagent compositions for immunoassays. Preferably, the cTnI conjugates are formed by conjugating a synthetic polymer, such as PEG, to the protein's pendant amine groups. Because cTnI's amine-containing lysine residues are not located in the cardiac-specific N-terminal portion of the protein, conjugation to PEG does not appreciably affect the ability of the cTnI to bind to cardiac-specific anti-cTnI antibodies.

In another embodiment, the present invention relates to a method of stabilizing individual subunits of multisubunit complexes by providing a solution of the individual subunit, adding a multifunctional crosslinking agent to activate the subunit, then simultaneously or subsequently adding a polymer to effect conjugation of the subunit to the polymer via the crosslinking agent. This method of stabilization is particularly preferred for conjugating serum proteins such as albumin to cTnT via glutaraldehyde, although it is also useful for other combinations of polymers and subunits as described herein, as well as with other multifunctional crosslinking agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow diagram which shows the activation of PEG and coupling to cTnI.

Figures 2A and 2B are bar graphs which show the stability of free cTnI and cTnI-PEG conjugates, respectively, at 23°C.

Figure 3A and 3B are bar graphs which show the stability of free cTnI and cTnI-PEG conjugates, respectively, at 37°C.

Figures 4A, 4B and 4C are bar graphs which show the stability of protein conjugated cTnT during storage at 4°C or 23°C (4A and 4B, respectively) compared to unconjugated cTnT (4C).

MODES FOR CARRYING OUT THE INVENTION

The present invention relates to methods of stabilizing individual subunits of multisubunit protein complexes via conjugation to polymers. The term "stabilized" as used herein means that the conformation of the protein's subunit when suspended in a liquid medium is maintained for a longer period of time than the equivalent unconjugated or "free" subunit. Stabilization can be measured as a function of the subunit's ability to retain at least one biological activity when suspended in a liquid medium and stored for a period of time, relative to the subunit in its native state. For example, a subunit-polymer conjugate would be considered stabilized if it maintained a level of about 50% of at least one of its biological activities upon storage in a liquid medium, compared to a free subunit. "Biological activity" of a protein subunit can be any biological characteristic attributed at least in part to that particular subunit of a multisubunit complex, such as enzyme activity, ligand binding capacity, substrate recognition, antibody binding, nucleic acid binding and the like.

The hydrophobic interactions between subunits of multisubunit proteins (*i.e.* a protein's quaternary structure) lend stability to the complex, and also to the conformation of the individual subunits within the complex. Accordingly, isolation of a subunit from a multisubunit protein complex can cause many subunits to undergo conformational changes. For example, individual subunits of multisubunit *E.coli* enterotoxin have been

shown to lose ligand binding capacity when separated from the native complex. Tsuji *et al.*, *Microbiol. Immunol.* 39:817-819 (1995).

5 Since a protein's biological activity depends on its conformation, stability of a specific ligands such as antibodies that recognize the native conformation of a protein can be used to measure changes to that conformation.

10 Individual subunits of multisubunit proteins may be useful for a variety of purposes, such as for studying or exploiting the biological activities of each individual subunit in a multisubunit protein complex. For example, in immunoassays, where target specificity can be achieved only by targeting antibodies to a particular subunit of a multisubunit protein complex, the individual subunit is useful as a control reagent for the immunoassay.

#### **Cardiac Troponin I (cTnI)**

15 Troponin is a three-subunit complex of troponin I, T and C. The cardiac isoform of troponin I ("cTnI") is an ideal target for the study and diagnosis of acute myocardial infarction.

20 In one embodiment, the present invention relates to cTnI-polymer conjugates and methods for the preparation thereof. More particularly, this embodiment of the present invention involves conjugation of cTnI to polymers which act to prevent the uncomplexed cTnI subunit from losing its native conformation and thus losing its binding capacity for cTnI-specific antibodies during storage in a liquid medium.

25 The nucleic acid sequence of the gene which codes for cTnI and its amino acid sequence have previously been described by Armour *et al.* (*Gene* 131:287-292 (1993); GenBank Accession No. M64247, SEQ ID NO:1 and SEQ ID NO:2, respectively).

30 The term "cTnI" as used herein also intends fragments of cTnI that retain at least one epitope which is recognizable by a monoclonal or polyclonal antibody (or fragment thereof) that preferentially binds to cardiac isoforms of troponin I. Also included in the term "cTnI" are: polypeptides that have amino acid substitutions, deletions or insertions relative to the native or naturally occurring amino acid sequence of cTnI; and fusion proteins that contain all or a fragment of cTnI linked to another protein.

**Cardiac Troponin T (cTnT)**

Like cTnI, cTnT is also useful as a marker for the study of acute myocardial infarction. Thus, in another embodiment, the present invention relates to cTnT-polymer conjugates and methods for the preparation thereof. Similarly to cTnI, conjugation of cTnT to polymers also acts to maintain cTnT's native conformation.

The nucleic acid sequence of the gene which codes for cTnT and its amino acid sequence have previously been described by Townsend, *et al.*, *Genomics*, 21(2): 311-316 (1994); GenBank Accession No. X74819; SEQ ID NO.s 3 and 4.

The term "cTnT" as used herein also intends fragments of cTnT that retain at least one epitope which is recognizable by a monoclonal or polyclonal antibody (or fragment thereof) that preferentially binds to cardiac isoforms of troponin T. Also included in the term "cTnT" are: polypeptides that have amino acid substitutions, deletions or insertions relative to the native or naturally occurring amino acid sequence of cTnI; and fusion proteins that contain all or a fragment of cTnT linked to another protein.

**Isolating Individual Subunits**

Protein subunits such as cTnI and cTnT can be isolated from their natural source in human or animal tissue, or they can be prepared using recombinant techniques.

Recombinant techniques are well known in the art and involve isolation and/or synthesis of a polynucleotide encoding all or a fragment of the protein subunit and cloning into a suitable bacterial or eukaryotic expression vector. Such techniques are described in a variety of references, including but not limited to, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Vol. 1-3, eds. Sambrook *et al.* Cold Spring Harbor Laboratory Press (1989). After insertion of the vector into a suitable host cell and expression of the desired protein subunit, the protein subunit can be isolated and purified using known techniques. (See, for example, Scopes *Protein Purification: Principles and Practice*, 2nd Ed., Springer-Verlag, New York. (1987))

In particular, cTnI can be isolated from heart muscle and substantially purified as described in PCT Patent Application No. WO 94/27156. Therein, a procedure is given for affinity purification of cTnI on a troponin C affinity column. Human heart is trimmed of excess fat and valves and cut into 1 cm pieces at 4°C. The resulting tissue is homogenized

in one portion with 750 ml extraction buffer (75 mM Tris buffer, pH 8.0, containing 8 M urea, 15 mM mercaptoethanol and 1 mM calcium chloride) at ambient temperature. The homogenate is centrifuged for 30 minutes at 7000 x g and the supernatant liquid is filtered through cheesecloth to remove particulate matter. Troponin C coupled to a solid support gel is equilibrated in extraction buffer, and the heart extract is added to the equilibrated troponin C-gel. The resulting suspension is allowed to stir for 80 minutes at ambient temperature and then centrifuged 20 minutes at 7000 x g. The pelleted gel is transferred to a column with extraction buffer. The column is washed at ambient temperature with a total of 700 ml of extraction buffer and the purified troponin I is then eluted from the column with elution buffer (75 mM Tris buffer, pH 8.0, containing 8 M urea, 15 mM mercaptoethanol, and 10 mM ethylenediamine tetraacetic acid).

The cTnT subunit can be prepared as generally described above for cTnI, or it can be commercially obtained from a variety of companies such as Research Diagnostics, Inc., Flanders, NJ; and HyTest, Turku, Finland.

Individual subunits of multisubunit protein complexes other than troponin can also be prepared as described above or by other known methods. In particular, individual subunits can be isolated from their natural source in biological samples by first separating the subunits, then using affinity chromatography with specific antibodies or other subunits of the complex.

#### Analyzing Individual Subunits

In the present invention, polymer conjugation is used to stabilize the conformation of individual protein subunits. Before proceeding with conjugation, it is preferable to first make sure the protein subunit preparation has the desired conformation. In particular, after isolation of individual subunits from multisubunit complexes, it is desirable to confirm that the isolation procedure did not adversely affect the conformation of the subunit. This can most easily be accomplished by determining whether the individual protein complex still has an acceptable level of the desired biological activity. For example, when the protein subunit is ultimately to be used as a control reagent in an immunoassay, antibody binding capacity should be determined. Testing the binding capacity of a protein for a particular antibody can be accomplished using known techniques. When the individual protein

subunit is cTnI/cTnT, cardiac-specific cTnI/cTnT antibodies are used to determine antibody binding capacity. "Cardiac-specific anti-cTnI/cTnT antibodies" refers to antibodies that have a substantially greater binding affinity for cTnI/cTnT than for other troponins or skeletal forms of cTnI/cTnT. Such antibodies to cTnI have been previously described. See, *inter alia*, Katus *et al.*, European Patent No. EP 394,819; Takahashi *et al.*, PCT Patent Application No. WO 96/10076; Larue *et al.*, *Clin. Chem.* 39:972-979 (1993); and Bodor *et al.*, *Clin. Chem.* 38:2203-2214 (1992). Antibodies which are specific for cTnT have also been described. See for example, Bodor, *et al.*, *Clinical Chemistry*, 43(3): 476-484 (1997), which describes a polyclonal goat anti-cTnT antibody (G136-C; Fortron BioScience, Morrisville, NC) which was developed against the N terminal amino acids 3-15 of human cTnT and which reportedly reacts <0.4% with skeletal isoforms. The N-terminal amino acid sequence is shown as follows:

(SEQ ID NO: 5)

Ile-Glu-Glu-Val-Val-Glu-Glu-Tyr-Glu-Glu-Glu-Gln

In addition, Muller-Bardorff, *et al.* (*Clinical Chemistry*, 43(3): 458-466 (1997)) describe a monoclonal antibody pair (M7 and M11.7) which can be used in a sandwich assay for cTnT which reportedly cross reacts <0.3% with skeletal isoforms.

20

### Polymers

Stabilization of individual protein subunits is achieved by conjugating the protein subunit to a polymer to prevent conformational changes of the protein subunit in the uncomplexed state and thus loss of its biological activity. The polymers of the present invention must have a high enough molecular weight to effectively stabilize the conformation of the protein subunit. Preferably, the molecular weight is between 100 to 200,000, more preferably between 1,000 and 40,000, and most preferably between 2,500 and 10,000.

The polymer may be already formed prior to conjugation to the protein subunit (i.e. the monomeric units comprising the polymer may already be covalently attached), or in the alternative, the polymer may be formed during conjugation by using monomeric units such

as monosaccharides, amino acids, or alkyl groups (C2 to C20 substituted or unsubstituted, branched or unbranched, saturated, partially saturated or unsaturated) which are "polymerized" (covalently attached to one another) during conjugation to the protein, or directly attached to the protein in their monomeric form. In any event, the term "polymer" and "polymer-protein conjugate" as used herein is intended to cover any of the aforementioned variations.

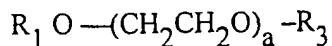
The polymer can be naturally occurring or synthetic. Examples of naturally occurring polymers include proteins, glycopeptides, polysaccharides such as dextran and lipids. In the case of cTnI, the polymer is preferably a synthetic polymer. Examples of synthetic polymers which are suitable for use in the present invention include, but are not limited to, polyalkyl glycols (PAG) such as PEG, polyoxyethylated polyols (POP) such as polyoxyethylated glycerol (POG), polytrimethylene glycol (PTG), polypropylene glycol (PPG), polyhydroxyethyl methacrylate, polyvinyl alcohol (PVA), polyacrylic acid, polyethyloxazoline, polyacrylamide, polyvinylpyrrolidone (PVP), polyamino acids, polyurethane and polyphosphazene. The synthetic polymers can also be linear or branched, substituted or unsubstituted, homopolymeric, or co-polymers of two or more different synthetic monomers.

The synthetic polymers of the present invention have the following generic structure:



where  $R_1$  and  $R_3$  are the same or different and are H,  $H_3C$ , OH,  $R_2$  or a reactive group (as described below); where  $R_2$  is a linear or branched substituted or unsubstituted alkyl group; where X is O (in which case the synthetic polymer may be a polyoxyalkylene) or X is  $NH(C=O)$  (in which case the synthetic polymer may be a polyamine), or X is absent (in which case the synthetic polymer may be a polyalkylene); and a is an integer between 1 and 1,000.

A preferred class of synthetic polymers are the polyethylene glycols given by the formula:



where  $R_1$ ,  $R_3$  and  $a$  are as described above. The term "polyethylene glycol" (PEG) includes both unsubstituted ( $R_1 = H$  and  $R_3 = OH$ ) as well as substituted polyethylene glycol.

5 Although hydrophilic polymers are preferred, it is also possible to use hydrophobic polymers, such as activated suberate or propionate derivatives, or mixtures of hydrophilic or hydrophobic polymers.

10 Naturally occurring proteins which are preferred for conjugation to cTnT are serum proteins, such as albumin. For convenience, the naturally occurring protein used for conjugation to cTnT can actually be a heterogeneous mixture of proteins, such as one which would be found in animal sera (for example, bovine or human), or in a synthetically produced serum substitute.

#### Polymer Activation

In order to conjugate the protein subunit to a polymer (which is either already polymerized or in its monomeric form at the time of conjugation), the polymer may first 15 need to be activated. "Activated" means the preliminary attachment of a reactive group onto the polymer. A polymer which has been activated, as well as a polymer which already contains at least one reactive group is referred to as being "active". Many synthetic polymers do not normally contain reactive groups which will react with a protein's pendant groups. For example, unsubstituted PEG has a hydroxyl group at each 20 end of the linear polymer chain, one or both of which must first be activated before conjugation to a protein. To prevent the potential for cross-linking, it is desirable to activate only one of PEG's two hydroxyl termini to form a "monofunctional" substituted PEG. This can be accomplished by blocking one of the two hydroxyl groups with a methoxy group to form monomethoxypolyethylene glycol (mPEG).

25 Techniques for activating polymers prior to protein conjugation are known to those skilled in the art. See, for example, Greg T. Hermanson, *Bioconjugate Techniques*, p. 605-619 (1996) Academic Press. For example, activation of hydroxyl groups in PEG or mPEG as well as in other natural or synthetic polymers, can be accomplished using trichloro-s-triazine (TsT; cyanuric acid). (See, *inter alia*, Abuchowski *et al.*, *J. Biol. Chem.* 30 252:3582-3586 (1977) and Abuchowski *et al.*, *J. Biol. Chem.* 252:3578-3581 (1977)). Another method of activating hydroxyl groups is through formation of an amine reactive

N-hydroxyl succinimidyl- (NHS) or p-nitrophenyl (Np) carbonate active ester. (See Zalipsky *et al.*, *Biotechnol. Appl. Biochem.* 15:100-114 (1992).)

Similar activation can be achieved when the hydroxyl-containing polymer is first reacted with a cyclic anhydride (succinic or glutaric anhydride) and then the formed carboxyl modified product is coupled with N-hydroxyl succinimide in the presence of carbodiimides resulting in succinimidyl succinate or glutarate type active esters. (See Abuchowski *et al.*, *Cancer Biochem. Biophys.* 7:175-186 (1984).)

A further method for activating a polymer's hydroxyl groups is through formation of an imidazolyl carbamate intermediate by reaction with N,N'-carbonyldiimidazole (CDI). The CDI-activated polymer reacts with amine groups of protein to form a stable N-alkyl carbamate linkage identical to that formed with succinimidyl carbonate chemistry described above. (See Beauchamp *et al.*, *Anal. Biochem.* 131:25-33 (1983).)

For additional methods on activating synthetic polymers, such as PEG, *see inter alia*, U.S. Patent No. 5,349,001; U.S. Patent No. 5,359,030; and U.S. Patent No. 5,446,090.

When the polymer to be conjugated to the protein subunit is a natural polymer such as a serum protein, it is generally preferred to activate the protein subunit by attachment of a multifunctional crosslinking agent as is more fully described in the following section.

#### 20 Polymer-Protein Conjugation

After activation of the polymer (if necessary), the polymer is conjugated to the individual protein subunit. In general, polymers can be covalently attached to proteins via pendant groups in the protein chain, such as primary amino groups, carboxyl groups, aromatic rings, or thiol groups, all of which may already be present, or can be added by preliminary chemical modification of the protein or by modifying a protein's amino acid sequence, using known molecular biology methods. One of the most frequently used and convenient methods involves attachment of the polymer to the protein's free amino groups in lysine residues ( $\epsilon$ -amino group) or to the protein's N-terminal amino acid ( $\alpha$ -amino group). This approach is preferred when the protein's amino groups are located in segments of the protein which are not crucial to maintaining the desired biological activity, such as is the case with cTnI. The discussion which follows outlines two representative

embodiments of the present invention. It is to be noted that the methodologies presented for cTnI are equally applicable for cTnT, and vice versa, as well as other protein subunits.

The ratio of polymer to protein to be used to carry out the conjugation depends on the characteristics (structure, size, charge, reactivity) of the polymer, as well as the characteristics (number and location of pendant groups, nature of the biological activity) of the individual subunit. It would be a matter of routine experimentation to determine the appropriate ratio by varying the ratio to optimize biological activity and conjugate stability.

A. cTnI-Polymer conjugates

There are three isoforms of troponin I: slow skeletal, fast skeletal and cardiac troponin I (herein referred to as "cTnI"). The three isoforms of troponin I in rabbits show about 40% sequence difference at the amino acid level, as described by Wilkinson and Grand *Nature*, 271:31-35 (1978). The nucleotide and amino acid sequences of human cTnI are given as SEQ ID NO:1 and SEQ ID NO:2, respectively. cTnI also contains a cardiac-specific 31 amino acid N-terminal sequence, which is completely missing from either of the two skeletal isoforms, as shown below:

(SEQ ID NO: 6)

Met-Ala-Asp-Gly-Ser-Ser-Asp-Ala-Ala-Arg-Glu-Pro-Arg-Pro-Ala-Pro-  
Ile-Arg-Arg-Arg-Ser-Ser-Asn-Tyr-Arg-Ala-Tyr-Ala-Thr-

Since this cardiac-specific segment contains no lysine residues, it is possible to conjugate polymers to amino groups located elsewhere on the protein without appreciably affecting the ability of the cTnI-PEG conjugate to bind to cardiac-specific anti-cTnI antibodies.

In the case of cTnI and PEG, the preferred ratio is between 1 and 20 moles of active PEG per mole of cTnI, more preferably between 6 and 16, and most preferably between 10 and 14. The resultant cTnI-PEG conjugate is considered stable if it retains at least 85% of its biological activity when stored at 4°C for a period of about 30 days. In the case of cTnI, "biological activity" refers to the ability of cTnI to be recognized by cTnI-specific antibodies. The cTnI-PEG conjugate is preferentially stored at -20°C to 23°C, more

preferably between about 2°C to about 8°C. The cTnI-PEG conjugate may also be lyophilized or stored under an inert gas such as argon, nitrogen, etc.

5           B. cTnT-Polymer Conjugates

In addition to conjugations which involve preactivation of the polymer, it is also possible to effect conjugation via a crosslinking agent. The crosslinking agent is preferably multifunctionally active and more preferably difunctionally active, which means that it contains more than one ("multi-") or two ("di-") reactive groups. The crosslinking agent can be one of the synthetic polymers described above which has been activated to contain two or more reactive groups, which can then be used as a linker to conjugate the protein subunit to another synthetic or natural polymer.

10           The crosslinking agent may also be a more conventional chemical crosslinking agent, including but not limited to, glutaraldehyde, formaldehyde, divinyl sulfane, carbodiimides, epoxides and imidazole. In the case of cTnT, glutaraldehyde is preferred.

15           **Protein-Polymer Conjugate Compositions**

Protein-polymer compositions can be formulated by diluting, dissolving or concentrating and rediluting the protein-polymer conjugate in a suitable liquid medium, such as water, aqueous solvents, serum, or mixtures thereof. Excipients can also be added to the liquid medium to further optimize the formulation. For example, cyclodextrin or other carbohydrates can be added to the protein-polymer conjugate to inhibit the rate of intermolecular aggregation during storage in the liquid medium.

20           Other optional components include, but are not limited to, casein, albumin, gelatin or other proteins. Buffers can be added to stabilize the pH of the liquid medium. Protease inhibitors such as phenyl methyl sulfonyl fluoride, leupeptin, pepstatin may also be included. Preservatives such as Amphotericin B, cycloheximide, chloramphenicol, bacitracin, gentamycin, chlorohexamide, sodium azide, trimethoprim, sulphomethaxazole may also be included. Additionally, reducing agents such as glutathione may be added.

25           It may also be desirable to sterilize the protein-polymer conjugate composition after formulation, such as by means of filtration. Filtration may be accomplished by passing the protein-polymer conjugate composition through a suitable size control device, such as a

filter, molecular sieves, resins, hollow fibers, and spiral cartridge exclusions. Preferably, a 0.2 micron aseptic filter is used.

Preferably, the liquid medium is mammalian serum, more preferably a mixture of bovine and human serum, which has been filter sterilized, and which also contains additional albumin, carbohydrates, and antimicrobial agents. For a description of such a "modified" human serum, see U.S. Patent No. 5,556,788. The serum used in the examples which follow were modified.

### EXAMPLES

10

#### EXAMPLE I

##### Conjugation of cTnI to PEG

cTnI from Scripps Laboratories (La Jolla, California) was dissolved in water at 4°C to a concentration of 1 mg/ml. To 50 µl of this cTnI solution, an equal volume of conjugation buffer (0.1 M sodium phosphate, pH=7.5) was added, and the resultant mixture was kept on ice. Meanwhile, the activated polymer solution was prepared by dissolving 5 mg methoxypolyethylene glycol p-nitrophenyl carbonate (mPEG-ONp, avg. MW=5,000) in 1 ml of ice-cold conjugation buffer. 42 µl of activated polymer solution (200 fold molar excess) were added to the buffered cTnI solution, and the mixture was incubated for 18 hours at 4°C with slow end-to-end mixing. The cTnI-PEG conjugate thus formed was frozen and stored at -20°C until use.

25

#### EXAMPLE II

##### Stability of cTnI-PEG Conjugates

The stability of the cTnI-PEG conjugates prepared according to Example I was compared to that of unconjugated cTnI. Both the conjugated and unconjugated cTnI preparations were diluted in a liquid medium of 50% human and 50% bovine serum, and stored at various temperatures. At the indicated times, samples were removed and

analyzed as described in Example III. The results are shown in Figures 2 and 3 as a percentage of the concentration of cTnI at a given time compared to the initial concentration (100%). The results depicted in Figure 2 indicate that, while unconjugated cTnI stored at 23°C for 20 days retains only about 20% of its initial antibody binding capacity, the cTnI-PEG conjugate retains essentially 100% activity when stored under the same conditions. Similar results are obtained when the unconjugated cTnI and the cTnI-PEG conjugates are stored at 37°C as depicted in Figure 3. Storage at 4°C of unconjugated cTnI typically results in the loss of at least 25% of its original antibody binding activity, while the cTnI-PEG conjugate retains 100% of its activity.

10

EXAMPLE III  
Measuring Antibody Binding Capacity

Antibody binding capacity is measured using either of the two commercially available assays for the quantitative determination of cTnI levels in serum and plasma according to the manufacturer's specifications. These assays are: the Stratus® cardiac Troponin-I fluorometric enzyme immunoassay (Baxter Diagnostics Inc./Dade International Inc., Miami, Florida); and the OPUS® Troponin I assay (Behring Diagnostics Inc., Westwood, Massachusetts).

20

EXAMPLE IV  
Conjugation of cTnT to Serum Proteins

Glutaraldehyde was added to a 400 mg/mL solution of cTnT from human heart tissue (HyTest, Turku, Finland) to a concentration of 0.1%. After mixing well and refrigerating, the glutaraldehyde activated cTnT was diluted 1:10 in either human serum or bovine serum. It is hypothesized that albumin, being the most prevalent protein which is present in the serum, is conjugated to the cTnT via the glutaraldehyde.

EXAMPLE VStability of cTnT-Serum Protein Conjugates

5        Stability studies were performed as described above for cTnI, and the results are shown in Figures 4A-C. As depicted, the serum conjugated cTnT is stable for more than 6 months at either 4°C or 23°C.

10      Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in the field of protein chemistry are intended to be within the scope of the following claims. All publications, patents, and patent applications cited in this specification are incorporated herein by reference as if each such publication, patent or patent application were specifically and individually indicated to be incorporated herein by reference.

15

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANT: Davé, Kirti I.  
Botyanszki, Janos  
Sintar, Eva

(ii) TITLE OF THE INVENTION: Stabilized Conjugates of Uncomplexed Subunits of Multimeric Proteins

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: MORRISON & FOERSTER  
(B) STREET: 755 PAGE MILL ROAD  
(C) CITY: Palo Alto  
(D) STATE: CA  
(E) COUNTRY: USA  
(F) ZIP: 94304-1018

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 35,053  
(C) REFERENCE/DOCKET NUMBER: 32260-20004.20

(ix) TELECOMMUNICATION INFORMATION:

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(C) TELEX: 706141

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 633 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence  
(B) LOCATION: 1...630  
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCG CAC GCC AAG AAA AAA TCT AAG ATC TCC GCC TCG AGA AAA TTG CAG Pro His Ala Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln 35 40 45	144
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CAG CCG CTG GAG TTG GCC GGG CTG GGC TTC GCG GAG CTG CAG GAC TTG Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu 85 90 95	288
TGC CGA CAG CTC CAC GCC CGT GTG GAC AAG GTG GAT GAA GAG AGA TAC Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr 100 105 110	336
GAC ATA GAG GCA AAA GTC ACC AAG AAC ATC ACG GAG ATT GCA GAT CTG Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu 115 120 125	384
ACT CAG AAG ATC TTT GAC CTT CGA GGC AAG TTT AAG CGG CCC ACC CTG Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu 130 135 140	432
CGG AGA GTG AGG ATC TCT GCA GAT GCC ATG ATG CAG GCG CTG CTG GGG Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly 145 150 155 160	480
GCC CGG GCT AAG GAG TCC CTG GAC CTG CGG GCC CAC CTC AAG CAG GTG Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val 165 170 175	528
AAG AAG GAG GAC ACC GAG AAG AAC CGG GAG GTG GGA GAC TGG CGC Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg 180 185 190	576
AAG AAC ATC GAT GCA CTG AGT GGA ATG GAG GGC CGC AAG AAA AAG TTT Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Phe 195 200 205	624
GAG AGC TGA Glu Ser 210	633

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 210 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein  
 (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
 20          25          30
Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
 35          40          45
Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
 50          55          60
Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
 65          70          75          80
Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
 85          90          95
Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
100         105         110
Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
115         120         125
Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
130         135         140
Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145         150         155         160
Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
165         170         175
Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
180         185         190
Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Phe
195         200         205
Glu Ser
210

```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 867 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence  
 (B) LOCATION: 1...864  
 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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 1           5           10          15

```

48

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GAA GAA GCA GCT GTT GAA GAG CAG GAG GAG GCA GCG GAA GAG GAT GCT
Glu Glu Ala Ala Val Glu Glu Gln Glu Glu Ala Ala Glu Glu Asp Ala
 20          25          30

```

96

```

GAA GCA GAG GCT GAG ACC GAG GAG ACC AGG GCA GAA GAA GAT GAA GAA
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```

144

35	40	45	
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CCA AAG CCC AGG TCG TTC ATG CCC AAC TTG GTG CCT CCC AAG ATC CCC Pro Lys Pro Arg Ser Phe Met Pro Asn Leu Val Pro Pro Lys Ile Pro 65 70 75 80			240
GAT GGA GAG AGA GTG GAC TTT GAT GAC ATC CAC CGG AAG CGC ATG GAG Asp Gly Glu Arg Val Asp Phe Asp Asp Ile His Arg Lys Arg Met Glu 85 90 95			288
AAG GAC CTG AAT GAG TTG CAG GCG CTG ATT GAG GCT CAC TTT GAG AAC Lys Asp Leu Asn Glu Leu Gln Ala Leu Ile Glu Ala His Phe Glu Asn 100 105 110			336
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AGA CGT CGG GCA GAG CGG GCC GAG CAG CAG CGC ATC CGG AAT GAG CGG Arg Arg Arg Ala Glu Ala Glu Gln Gln Arg Ile Arg Asn Glu Arg 130 135 140			432
GAG AAG GAG CGG CAG AAC CGC CTG CCT GAA GAG AGG GCT CGA CGA GAG Glu Lys Glu Arg Gln Asn Arg Leu Ala Glu Glu Arg Ala Arg Arg Glu 145 150 155 160			480
GAG GAG GAG AAC AGG AGG AAG GCT GAG GAT GAG GCC CGG AAG AAG Glu Glu Glu Asn Arg Arg Lys Ala Glu Asp Glu Ala Arg Lys Lys 165 170 175			528
GCT TTG TCC AAC ATG ATG CAT TTT GGG GGT TAC ATC CAG AAG CAG GCC Ala Leu Ser Asn Met Met His Phe Gly Gly Tyr Ile Gln Lys Gln Ala 180 185 190			576
CAG ACA GAG CGG AAA AGT GGG AAG AGG CAG ACT GAG CGG GAA AAG AAG Gln Thr Glu Arg Lys Ser Gly Lys Arg Gln Thr Glu Arg Glu Lys Lys 195 200 205			624
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AG

867

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 288 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Glu Glu Ala Ala Val Glu Glu Gln Glu Glu Ala Ala Glu Glu Asp Ala
   20          25          30
Glu Ala Glu Ala Glu Thr Glu Glu Thr Arg Ala Glu Glu Asp Glu Glu
   35          40          45
Glu Glu Glu Ala Lys Glu Ala Glu Asp Gly Pro Met Glu Glu Ser Lys
   50          55          60
Pro Lys Pro Arg Ser Phe Met Pro Asn Leu Val Pro Pro Lys Ile Pro
   65          70          75          80
Asp Gly Glu Arg Val Asp Phe Asp Asp Ile His Arg Lys Arg Met Glu
   85          90          95
Lys Asp Leu Asn Glu Leu Gln Ala Leu Ile Glu Ala His Phe Glu Asn
  100          105         110
Arg Lys Lys Glu Glu Glu Leu Val Ser Leu Lys Asp Arg Ile Glu
  115          120         125
Arg Arg Arg Ala Glu Arg Ala Glu Gln Gln Arg Ile Arg Asn Glu Arg
  130          135         140
Glu Lys Glu Arg Gln Asn Arg Leu Ala Glu Glu Arg Ala Arg Arg Glu
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Gln Thr Glu Arg Lys Ser Gly Lys Arg Gln Thr Glu Arg Glu Lys Lys
  195          200         205
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  225          230         235         240
Tyr Asn Leu Glu Ala Lys Phe Asp Leu Gln Glu Lys Phe Lys Gln
  245          250         255
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## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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1                   5                   10

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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1                   5                   10                   15  
Ala Pro Ile Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr  
20                   25                   30

CLAIMS

We claim:

1. A method of stabilizing an individual subunit of a multisubunit protein complex in liquid medium comprising the steps of:
  - 5 a) providing a solution of said individual subunit; and
  - b) mixing said individual subunit solution with an active polymer for a time sufficient and under conditions suitable to form a stabilized individual subunit-polymer conjugate.
- 10 2. The method according to claim 1, wherein the individual subunit is cTnI.
3. The method according to claim 1, wherein the polymer is a naturally occurring polymer.
- 15 4. The method according to claim 1, wherein the polymer is a synthetic polymer.
5. The method according to claim 4, wherein the synthetic polymer is PEG.
- 20 6. The method according to claim 4, wherein the synthetic polymer is selected from a group consisting of polyalkylene glycols, polyoxyethylated polyols, polyvinylpyrrolidone, polyhydroxyethyl methacrylate, polyvinyl alcohols, and polyurethane.
- 25 7. The method according to claim 1, wherein the polymer has an average molecular weight between 100 and 200,000.
8. The method according to claim 1, wherein the polymer has an average molecular weight between 1,000 and 40,000.
- 30 9. The method according to claim 1, wherein the polymer has an average molecular weight between 2,500 and 10,000.

10. The method according to claim 5, wherein the PEG has an average molecular weight between 2,500 and 10,000.

5 11. The method according to claim 5, wherein the PEG has an average molecular weight of about 5,000.

12. The method according to claim 1, wherein the individual subunit is cTnI and the polymer is PEG.

10 13. The method according to claim 12, wherein the PEG is conjugated with the cTnI in a molar ratio of about 1:1 to 20:1 PEG to cTnI.

14. The method according to claim 5, wherein the PEG is monomethoxy-PEG  
15 p-nitrophenyl carbonate.

15. The method according to claim 2, wherein the cTnI is a fragment of cTnI capable of being recognized by a cardiac-specific cTnI antibody.

20 16. The method of claim 1, wherein the polymer is in its monomeric form when mixed with said individual subunit.

25 17. A stabilized individual subunit-polymer conjugate composition comprising:  
a) a conjugate of an individual subunit of a multisubunit protein complex  
and a polymer; and  
b) a liquid medium.

18. The composition according to claim 16, wherein the liquid medium is mammalian serum.

30 19. The composition according to claim 16, wherein the liquid medium is a mixture of human serum and bovine serum.

20. The composition of claim 16, wherein the individual subunit is cTnI and the polymer is PEG.

5 21. The composition according to claim 16, wherein the stabilized individual subunit-polymer conjugate is more stable in said liquid medium than an equivalent free individual subunit.

10 22. A method of stabilizing an individual subunit of a multisubunit protein complex in liquid medium comprising the steps of:

- a) providing a solution of said individual subunit;
- b) adding a multifunctional crosslinking agent to said solution to form an active individual subunit; and
- c) mixing said active individual subunit with a polymer for a time sufficient and under conditions suitable to form a stabilized individual subunit-polymer conjugate.

15 23. The method according to claim 22, wherein the individual subunit is cTnT.

20 24. The method according to claim 22, wherein the polymer is a naturally occurring polymer.

25 25. The method according to claim 24, wherein the natural polymer is albumin.

26. The method according to claim 25, wherein the albumin is selected from the group consisting of human serum albumin, bovine serum albumin, and a mixture of human and bovine serum albumin.

30 27. The method of claim 22, wherein the multifunctional crosslinking agent is glutaraldehyde.

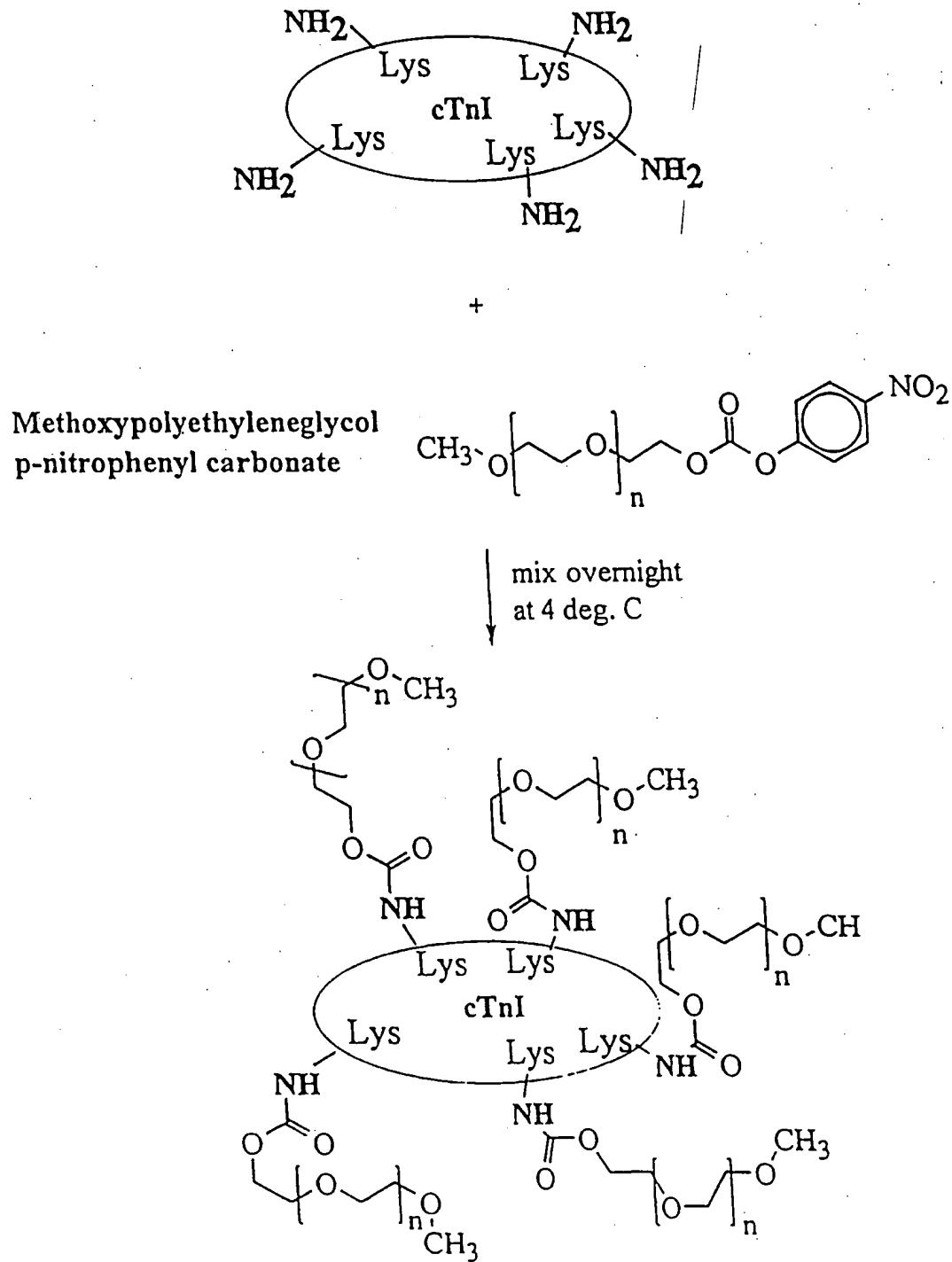
28. The method of claim 22, wherein the multifunctional crosslinking agent is a polyethylene glycol.

29. A covalent protein-polymer conjugate, wherein the protein is free cTnI and  
5 the polymer is polyethylene glycol.

30. A covalent protein-polymer conjugate, wherein the protein is free cTnT and  
the polymer is a serum protein.

1/4

FIG. 1

Conjugation of cTnI to PEG

2/4

Fig. 2A

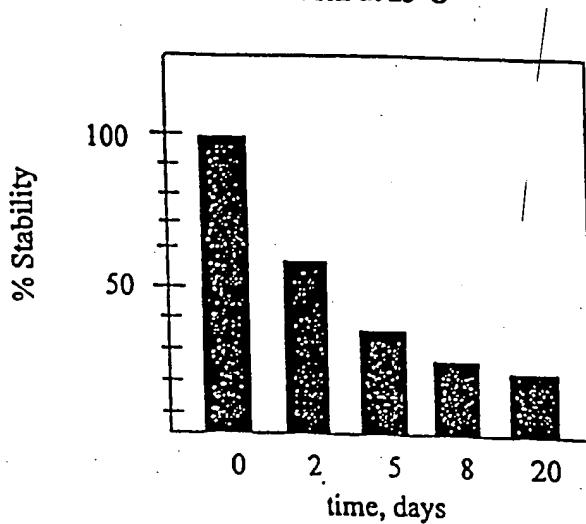
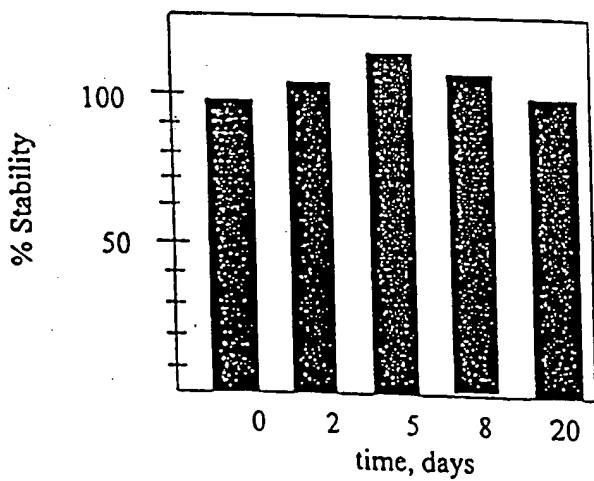
Stability of free  
cTnI at 23°C

Fig. 2B

Stability of cTnI - PEG  
Conjugate at 23°C

3/4

Fig. 3A

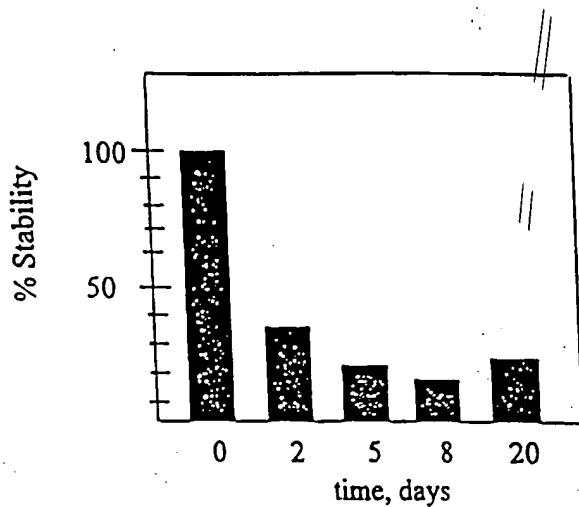
Stability of free  
cTnI at 37°C

Fig. 3B

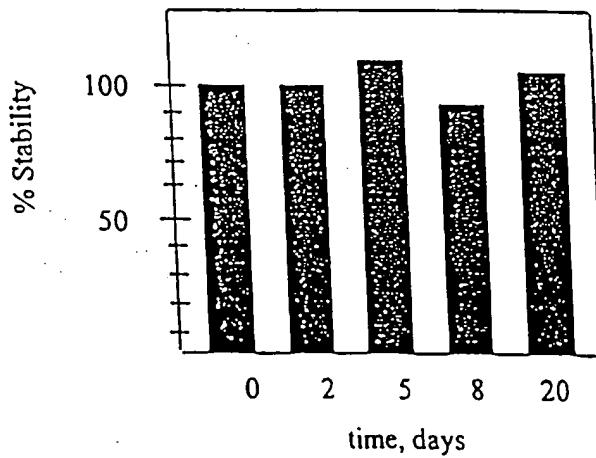
Stability of cTnI - PEG  
Conjugate at 37°C

FIG. 4A 4/4

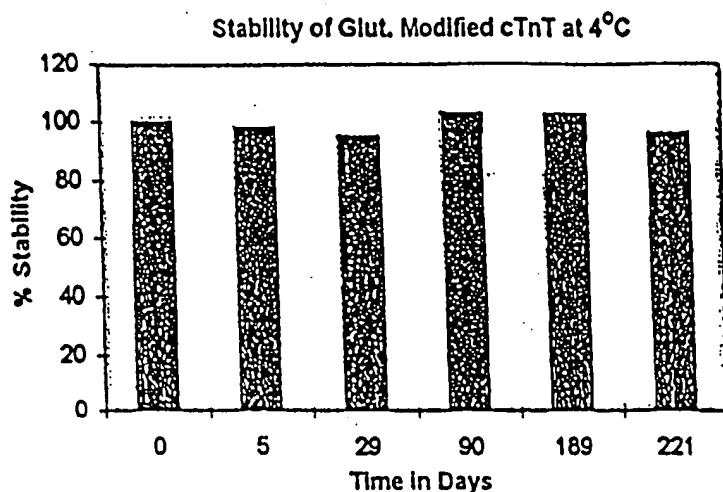


FIG. 4B

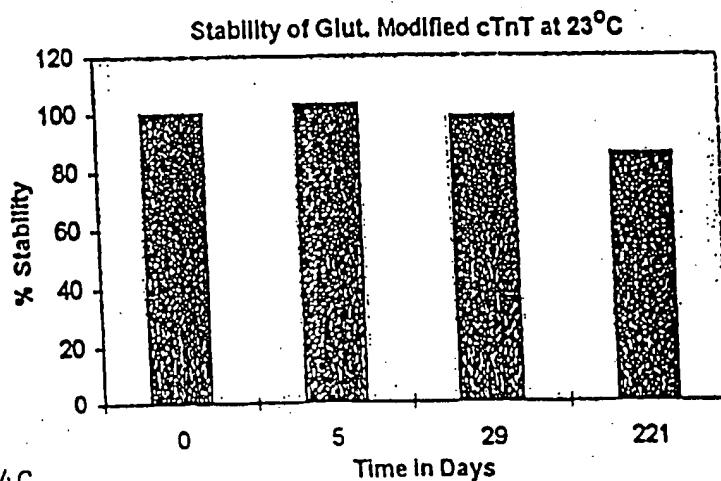
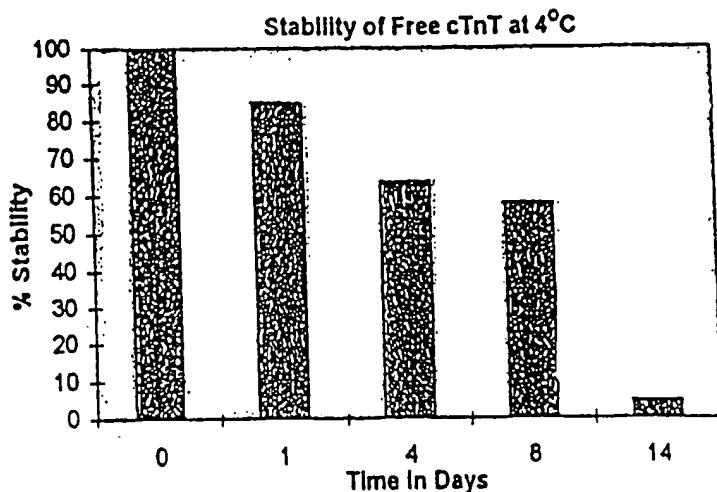


FIG. 4C





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:  A61K 47/48		A3	(11) International Publication Number: <b>WO 98/16255</b>  (43) International Publication Date: 23 April 1998 (23.04.98)						
<p>(21) International Application Number: PCT/US97/18368</p> <p>(22) International Filing Date: 15 October 1997 (15.10.97)</p> <p>(30) Priority Data:</p> <table> <tr> <td>08/730,111</td> <td>15 October 1996 (15.10.96)</td> <td>US</td> </tr> <tr> <td>Not furnished</td> <td>15 October 1997 (15.10.97)</td> <td>US</td> </tr> </table> <p>(71) Applicant (<i>for all designated States except US</i>): NAVIX, INC. [US/US]; 542 Flynn Road, Camarillo, CA 93012 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): DAVÉ, Kirti, I. [IN/US]; 2533 Vistawood Circle #24, Thousand Oaks, CA 91362 (US). BOTYANSZKI, Janos [HU/US]; 813 Paseo Camarillo #490, Camarillo, CA 93010 (US). SINTAR, Eva [HU/US]; 2531 Bogart Street, Camarillo, CA 93010 (US).</p> <p>(74) Agents: AXFORD, Laurie, A. et al.; Morrison &amp; Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).</p>		08/730,111	15 October 1996 (15.10.96)	US	Not furnished	15 October 1997 (15.10.97)	US	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b></p> <p><i>With international search report.</i></p> <p><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 4 June 1998 (04.06.98)</p>	
08/730,111	15 October 1996 (15.10.96)	US							
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<p>(54) Title: STABILIZED CONJUGATES OF UNCOMPLEXED SUBUNITS OF MULTIMERIC PROTEINS</p> <p>(57) Abstract</p> <p>The present invention provides methods for preparing, and compositions comprising, stabilized protein-polymer conjugates. More particularly, the present invention relates to the stabilization of individual subunits of multisubunit proteins complexes by conjugation to polymers. Such conjugation acts to stabilize the individual subunit in its native conformation in liquid medium, which in turn acts to stabilize its biological activity.</p>									

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/18368

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP 0 752 426 A (BAYER AG) 8 January 1997 see column 1, line 22 - column 2, line 45; claims 2-5 see column 3, line 15-25 ---	1-30
X	EP 0 650 053 A (BOEHRINGER MANNHEIM GMBH) 26 April 1995  see page 4 see page 6, line 25-55; figures 3,4; examples 4,5 ---	1,3,7, 15-19, 21-28,30
Y	US 4 970 156 A (AVRAMEAS STATIS ET AL) 13 November 1990  see claims 1,8,10,17,27 see page 4, line 66 - page 5, line 35 see page 14, line 54 - page 15, line 5 ---	1-3, 15-19, 21-27,30
	-/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

27 March 1998

Date of mailing of the international search report

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Name and mailing address of the ISA

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/18368

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZALIPSKY S: "CHEMISTRY OF POLYETHYLENE GLYCOL CONJUGATES WITH BIOLOGICALLY ACTIVE MOLECULES" ADVANCED DRUG DELIVERY REVIEWS, vol. 16, no. 2/03, 1995, pages 157-182, XP002037428 see page 160, column 1; figures 1,2 see page 161, column 2, paragraph 2; table 1 see page 164, column 2, paragraph 3 ---	1,2, 4-23,28, 29
Y	WO 96 27661 A (DADE INT INC) 12 September 1996 see page 9, line 22 - page 10, line 10; figures 1-5 see page 13, line 25 - page 14, line 10; claims 3,11; examples 4-7,10	1-30
L	see page 5, line 22 - page 6, line 10 ---	
A	FISHER, D. ET AL: "PEG-protein constructs for clinical use" PERSPECT. PROTEIN ENG. COMPLEMENTARY TECHNOL., COLLECT. PAP., INT. SYMP., 3RD, 1995, 223-226, XP002060507 see page 224; figure 1 -----	1-30
2		

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 97/18368

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 1,3,4

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

In view of the large number of compounds, which are defined by the general definition in the independent claims, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application (see Guidelines, Part B, Chapter III, paragraph 3.6).

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/US 97/18368

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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